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Fusobacterium nucleatum Outer Membrane Proteins Fap2 and RadD Induce Cell Death in Human Lymphocytes▽

Christopher W. Kaplan,[1]† Xiaoyuan Ma,[2] Avina Paranjpe,[2]‡ Anahid Jewett,[2] Renate Lux,[2] Susan Kinder-Haake,[2] and Wenyuan Shi[1,2,3,4]*

Molecular Biology Institute[1] and Schools of Dentistry,[2] Oral Biology,[3] and Microbiology, Immunology and Molecular Genetics,[4] University of California, Los Angeles, California 90095

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Bacterially induced cell death in human lymphocytes is an important virulence factor for pathogenic bacteria. Previously discovered mechanisms of bacterially induced cell death are predominantly based on the transfer of bacterial proteins to the target host cell, such as the toxins secreted through type I, II, and VI secretion systems or effector proteins injected through type III, IV, and Vb secretion systems. Here, we report a mechanism employed by the Gram-negative oral pathogen *Fusobacterium nucleatum* for cell death induction of human lymphocytes via two outer membrane proteins (OMPs), Fap2 and RadD, which share regions homologous to autotransporter secretion systems (type Va secretion systems). Genetic and physiological studies established that inactivation of the two OMPs led to significantly reduced ability to trigger cell death in Jurkat cells, while the corresponding double mutant was almost completely attenuated. Additional biochemical and molecular analyses demonstrated that cell-free *F. nucleatum* membranes are sufficient to induce cell death in Jurkat cells, suggesting that no active process or effector protein transfer was necessary to induce eukaryotic cell death.

Bacterially induced cell death in human lymphocytes is a key virulence factor which allows bacteria to fight the host immune system and establish pathogenesis (8). These cell death events are achieved via a host of different virulence factors, some of which are secreted through a range of bacterial secretion pathways. Currently, six specialized secretion pathways (types I, II, III, IV, V, and VI) have been found to mediate cell death in host cells via the active transfer of bacterial effector proteins to the host cells. The type I secretion system (T1SS) is used by many bacterial species to secrete apoptosis-inducing proteins, exemplified by the pore-forming RTX toxin HylA, found in uropathogenic *Escherichia coli* (14, 26, 44). The prevalent T2SSs have been demonstrated in certain species to be involved in virulence (3). A number of plant pathogens, including *Erwinia chrysanthemi*, secrete proteins via the T2SS to cause tissue destruction that leads to cell death (17). T3SSs are one of the most common virulence factors in Gram-negative pathogenic bacteria. T3SSs function by injecting effector proteins into host cells, where they induce apoptosis (4). *Shigella flexneri* employs one of the best-studied examples of the T3SS to inject IpaB into the host cell, initiating apoptotic events through activation of caspase-1 (13, 46). In *Legionella pneumophilia*, the Dot/Icm T4SS is essential to trigger the apoptotic pathway through activation of caspase-3 (39, 45). The type Va autotransporter VacA is an important virulence factor in *Helicobacter pylori* that is thought to induce apoptosis in host cells via secretion of its N-terminal domain (25). The most recently described secretion system is the T6SS. The T6SS is used by *Vibrio cholerae* to transport proteins that are proposed to puncture the cell membrane into macrophages (37).

The Gram-negative anaerobic bacterium *Fusobacterium nucleatum* is a prevalent member of the oral microbial community and has been associated with periodontal disease. The opportunistic nature of *F. nucleatum* pathogenesis has been concluded from its increased abundance in the oral cavity during periodontal disease (2, 33) and its ability to interfere with the immune system via unknown immunosuppressive factors (2, 6, 29, 30, 41, 42). The genomes of several *F. nucleatum* strains revealed a curious absence of genes encoding any obvious virulence factors such as T3SS or T4SS (19, 20). However, the genomes also disclosed that *F. nucleatum* possesses a conserved family of large outer membrane proteins (OMPs), varying in predicted size from 200 to 400 kDa, that were classified as type Va secretion systems (T5SSs) or autotransporters (7, 19, 20).

In previous studies, we discovered that part of the immunosuppressive ability of *F. nucleatum* may be derived from its ability to induce cell death in Jurkat cells, possibly through activating their apoptotic pathways (16). In particular, this study indicated that a heat-labile cell surface protein present on *F. nucleatum* was responsible for this important virulence factor (16). To identify these virulence factors, we created an *F. nucleatum* gene library in *E. coli* and screened the cells for their ability to induce cell death in Jurkat cells. Here we report the identification of two T5SS proteins, Fap2 and RadD, as the molecular components carrying out this task. Further analyses demonstrated that cell-free membrane preparations induced cell death in Jurkat cells via a mechanism that does not appear

* Corresponding author. Mailing address: UCLA School of Dentistry, CHS 20-118, 10833 Le Conte Avenue, Los Angeles, CA 90095. Phone: (310) 825-8356. Fax: (310) 794-7109. E-mail: wenyuan@ucla.edu.

† Present address: Endodontics, University of Washington, Seattle, WA 98195.

‡ Present address: C3 Jian, Inc., Inglewood, CA 90301.

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to require an active process or translocation of effector proteins.

MATERIALS AND METHODS

Cell lines, bacterial strains, and culture conditions. Jurkat cells (immortalized human lymphocytes) were maintained in RPMI 1640 supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 1% penicillin-streptomycin, and 10% fetal calf serum (Life Technologies, Grand Island, NY). *Fusobacterium nucleatum* strains PK1594 and ATCC 23726 were grown in Columbia broth and incubated anaerobically ($N_2/H_2/CO_2$, 90:5:5) at 37°C. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) cells used for cloning were grown aerobically in Luria-Bertani medium at 37°C.

Creation of gene inactivation mutants. *F. nucleatum* ATCC 23726 genes NP_603161.1, NP_603291.1, NP_604343.1, NP_602353.1, NP_602381.1, NP_602692.1, NP_602832.1, and NP_602843.1 were inactivated by single homologous recombination as previously described (9, 22, 23). Briefly, gene fragments were amplified by PCR using appropriate primers and ligated into pHS31 (22) for single gene mutants and pHS19 (9) when constructing double gene mutants. Plasmids were then used to transform *E. coli* TOP10 cells. Cells were plated on LB medium containing either 25 μg/ml chloramphenicol for pHS31 transformants or 100 μg/ml erythromycin for pHS19 transformants. PCRs were carried out with the following reagents in 50-μl reaction volumes: template DNA, 10 ng; 1× *Taq* buffer; deoxynucleoside triphosphates, 3×10^{-5} mmol; bovine serum albumin, 4×10^{-2} μg; $MgCl_2$, 1.75×10^{-4} mmol; primers, 10^{-5} mmol each (21); *Taq* DNA polymerase, 2.5 U. Reaction temperatures and cycling for samples were as follows: 95°C for 1 min, then 35 cycles of 92°C for 1 min, 55°C for 30 s, and 72°C for 2 min, followed by 72°C for 10 min. Products were visualized by agarose gel electrophoresis. The resulting integration plasmids were confirmed by restriction analysis and PCR amplification using each insert's primers (21). *F. nucleatum* ATCC 23726 was transformed with the individual gene inactivation constructs by electroporation and plated on Columbia blood agar containing 5 μg/ml thiamphenicol for pHS31 transformants and/or 0.4 μg/ml clindamycin for pHS19 transformants. Insertional mutations were confirmed by PCR using upstream and downstream primers and by liquid chromatography/tandem mass spectrometry (LC/MS/MS) identification of proteins isolated from SDS-PAGE bands. LC/MS/MS was performed at the UCLA W. M. Keck Proteomic Center.

Bacterial membrane isolation. Bacterial cells were pelleted by centrifugation and resuspended in buffer (20 mM sodium phosphate buffer with 150 mM NaCl, pH 7.2) containing Complete protease inhibitor cocktail (Roche, Indianapolis, IN). Cells were lysed via French press three times at 12,000 lb/in^2. Unbroken cells were removed by centrifugation (10,000 × *g* at 4°C for 10 min). Membranes were pelleted by ultracentrifugation (150,000 × *g* at 4°C for 60 min), resuspended in 2 ml buffer, and stored at −80°C until use.

Cell death assay. Induction of cell death in Jurkat cells was determined by flow cytometry using propidium iodide staining (16) or by annexin V-fluorescein isothiocyanate (FITC) assay according to the manufacturer's protocol (Bender Medsystems, Burlingame, CA). Briefly, in 2 ml of RPMI 1640, 1×10^6 Jurkat cells/ml were infected with an overnight culture of *F. nucleatum* cells (multiplicity of infection [MOI] of 100:1, i.e., 1×10^8 cells/ml) or *F. nucleatum* cell membranes (100 μg/ml) and incubated for 18 h at 37°C (5% CO_2) in RPMI 1640. Cells were washed in phosphate-buffered saline (PBS) and centrifuged at 200 × *g* for 5 min. Supernatant was aspirated from the cell pellet, resuspended in 100 μl of annexin V-FITC (1 μg/ml in HEPES buffer with 1.8 mM $CaCl_2$), and incubated for 5 to 10 min at room temperature in the dark. After incubation, 1 ml of HEPES containing 10 μg/ml propidium iodide was added to the cells and cells were analyzed by flow cytometry. Results are representative of at least 3 individual experiments. Results obtained with annexin V-FITC and propidium iodide were similar.

Transcriptional analysis. Three micrograms of total RNA was used for cDNA synthesis using Stratascript RT (Stratagene, La Jolla, CA) according to the manufacturer's protocol. For real-time reverse transcription-PCR (RT-PCR), SYBR green (Bio-Rad, Hurcules, CA) was used for fluorescence detection with the iCycler (Bio-Rad) real-time PCR system according to the manufacturer's protocol as previously described (32) with the primers listed in Table 1. All values presented represent the ratios of expression compared to 16S rRNA expression. Transcription levels of an inducible fructose kinase gene homolog were used as the baseline control for gene expression.

Statistical analysis. Student's *t* test was performed using Microsoft (Redmond, WA) Excel 2007.

TABLE 1. Primers used for real-time PCR in this study

Primer	Sequence	Strain
Fap2rt-F	GGGGAAATAGGTCGTTCTGC	PK1594
Fap2rt-R	CCAACCCCAACACTTTCATC	PK1594
FruK-F	CTGTTGGTGCAGGAGATTCA	PK1594
FruK-R	CACAAGCCACTGCAAACCTA	PK1594
Fnp16Srt-F	TAAGGGGTTGTGGGACAGAG	PK1594
Fnp16Srt-R	ACTTTCACCTTCTGCGGTTC	PK1594
Fn1448rt-F	TGAAGAAGAAATGATGAATGAAGG	ATCC 23726
Fn1448rt-R	CTTTGAGGCAAAAACTTGTGT	ATCC 23726
Fn1449rt-F	AAAATTGGAGCAACAGGAGGA	ATCC 23726
Fn1449rt-R	TTCAGAGGCAATAGCGACAAC	ATCC 23726
Fnn16Srt-F	GGTTAAGTCCCGCAACGA	ATCC 23726
Fnn16Srt-R	CATCCCCACCTTCCTCCTAC	ATCC 23726

RESULTS

Identification and transcriptional analysis of cell death-inducing genes. We previously reported that *F. nucleatum* induces cell death in human lymphocytes via activation of the apoptosis pathway and DNA fragmentation (16). Fixing the bacterial cells had no effect on their ability to induce cell death, but both heat and pronase treatment abolished this feature, implying that the corresponding virulence factor(s) was a heat-labile, pronase-sensitive cell surface protein(s). Additionally, a screen for *F. nucleatum* PK1594 genes that induce cell death in Jurkat cells identified *fap2* as a potential candidate (data not shown). Initial analysis of *fap2* indicated that it encodes a protein that has homology to the OMP family of *F. nucleatum* proteins (19) and type Va autotransporter secretion system proteins. The *fap2* gene encodes 3,692 amino acids, resulting in a very large OMP with a predicated molecular mass of 390 kDa. Structural analysis revealed parallel β-helix repeats along the entire sequence and an autotransporter β-domain (PF03797) at the N-terminal end, suggesting transport of this protein to the cell surface through a type Va secretion system or autotransporter (7, 12).

To determine the expression of *fap2* in *F. nucleatum*, we performed real-time PCR analysis. Transcriptional analysis in *F. nucleatum* PK1594 revealed a 3-fold induction of *fap2* expression during log phase growth or in the presence of Jurkat cells. Expression of *fap2* in stationary-phase cells or those grown on solid medium, compared to *fruK* expression, did not exceed low basal levels (Fig. 1).

Identification of Fap2 homologs. The current *F. nucleatum* genetic system has not been successful in transforming strain PK1594, requiring us to identify *fap2* homologs present in the transformable *F. nucleatum* strain ATCC 23726 (21, 22, 24). Our analysis of the *F. nucleatum* ATCC 23726 genome identified eight Fap2 homologs, all of which belonged to the OMP family of *F. nucleatum* proteins and had sequence similarities ranging from 39% to 71%. The recently described arginine-inhibitable adhesin RadD (21) showed the least similarity to Fap2 of PK1594, 39%, while the previously characterized AimI displayed about 48% homology (22). The protein from strain ATCC 23726 with the highest (71%) similarity to its counterpart in PK1594 was annotated Fap2 (*F. nucleatum* ATCC 23726 gene Fn1449). These results were similar to those described by other researchers for the sequenced *F. nucleatum* strain ATCC 25586 (7, 19). Structural and sequence analyses revealed that all of the homologs had similar parallel β-helix

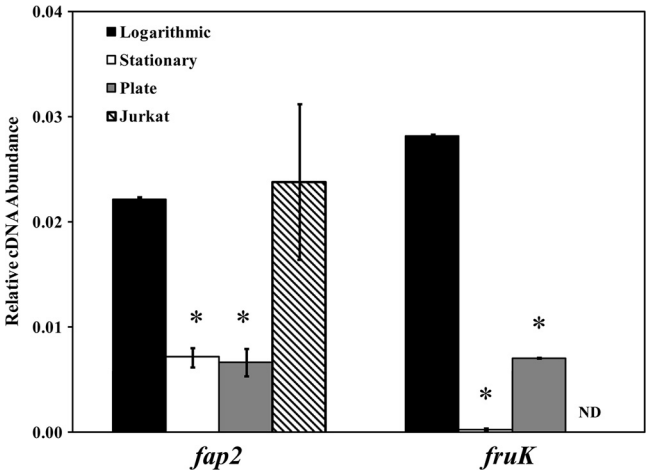

FIG. 1. Transcriptional analysis of *fap2* from *F. nucleatum* PK1594. Relative cDNA abundance of *fap2* was determined for cells grown in broth culture to logarithmic or stationary phase, on agar plates, or in the presence of Jurkat cells (4-h incubation) to logarithmic phase. $n = 3$. ND, not determined; *, $P \leq 0.01$ compared to logarithmic phase results.

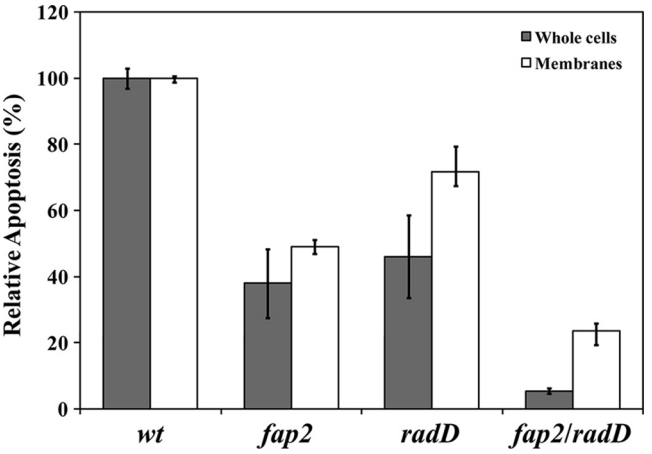

FIG. 2. Jurkat cell death induction by *F. nucleatum*. Cell death rates were quantified based on the percentage of annexin V-FITC-positive Jurkat cells in samples incubated with *F. nucleatum* whole cells or membranes. *F. nucleatum* ATCC 23726 cells and membranes induced cell death in ~75% and 41% of Jurkat cells, respectively, in the annexin V assay, and these numbers serve as the basis for the relative calculations presented. $n \geq 3$. $P \leq 0.01$ for all conditions compared to the wild-type control (*wt*).

structures and highly conserved C-terminal β-barrel autotransporter domains. Since the sequences of all Fap2 homologs identified in ATCC 23726 differed from that of Fap2 to various extents despite their structural similarity, we constructed gene inactivation mutants for each of the identified *fap2* homologs.

***Fusobacterium*-induced cell death is mediated by Fap2 and RadD.** The mutant derivatives lacking individual *fap2* homologs were characterized for their ability to induce cell death using an annexin V-FITC assay and quantitated by flow cytometry. Infection with mutant derivatives of ATCC 23726 lacking *fap2* or *radD* yielded a significant reduction of the death of Jurkat cells, with 38% and 46% of wild-type activity, respectively (Fig. 2). Since the *fap2* and *radD* mutants demonstrated only a partial decrease in cell death induction compared to the wild-type strain, we tested the possibility that each protein comprises a part of the cell death-inducing machinery of *F. nucleatum*. A double mutant lacking both *fap2* and *radD* function was constructed and found to have a 95% decrease in cell death induction in Jurkat cells compared to wild-type *F. nucleatum* (Fig. 2). In contrast, addition of the previously described cell death-related *aim1* mutation to either *fap2* or *radD* did not enhance the level of cell death-inducing ability in the respective double mutants, indicating that the corresponding gene product does not have a major involvement in Fap2- or RadD-related functions (data not shown).

Gene products of *fap2* and *radD* are large outer membrane proteins. To confirm the localization of the predicted outer membrane proteins Fap2 and RadD experimentally, we separated proteins present in isolated membrane fractions using SDS-PAGE. The insertional mutations generated in this study were expected to completely abolish surface expression of the protein products since the C-terminal autotransporter domain is required for membrane translocalization and anchoring (12). Absence of the respective proteins on the cell surface was confirmed for the individual *fap2* and *radD* mutant strains as well as the *fap2 radD* mutant lacking both genes by comparing their membrane preparations with the ones obtained from wild-type cells (Fig. 3). The missing bands were previously confirmed to correspond to Fap2 and RadD by LC/MS/MS analysis (21). While *radD* has been described as the last gene in a four-gene operon (21), genome analysis revealed the presence of an open reading frame (ORF; Fn1448) downstream of *fap2* that could be part of the same operon. To rule out the possibility that a polar effect on this ORF contributed to the observed deficiency of this mutant strain in inducing cell death in Jurkat cells, we performed real-time RT-PCR-based transcriptional analyses. Identical transcription levels of the downstream gene Fn1448 in wild-type and mutant cells indicated

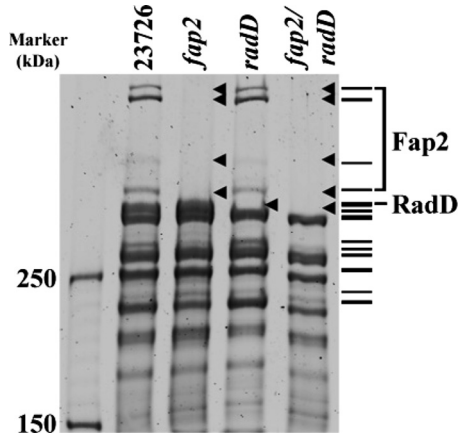

FIG. 3. Membrane preparation of *F. nucleatum* strains. Samples were boiled for 10 min in SDS gel loading buffer and resolved by 4% SDS-PAGE and Sypro Ruby staining. An illustration of the protein bands present in the wild-type strain is indicated on the right. Protein bands missing in mutant strains are indicated with arrowheads and labeled on the protein band illustration to the right. Molecular mass standards (marker) are indicated.

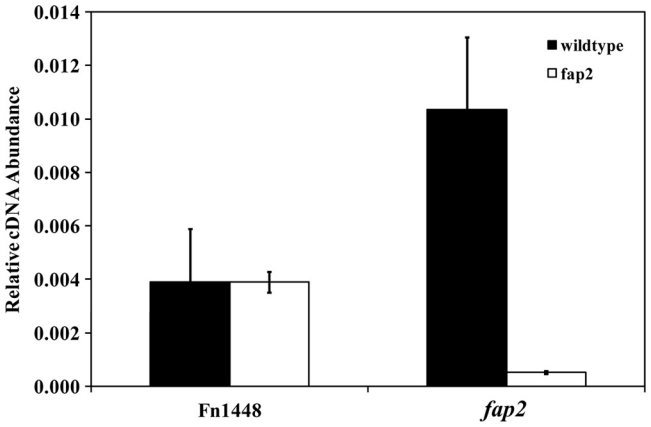

FIG. 4. Transcription of *fap2* and its downstream ORF, Fn1448, in wild-type *F. nucleatum* and the mutant derivative lacking *fap2*. *F. nucleatum* cells were grown to mid-exponential-growth phase to ensure *fap2* expression. *fap2*, but not Fn1448, expression in the mutant was significantly different ($P \leq 0.01$) from that in the wild type. $n = 3$.

that there was no polar effect and that the two genes were transcribed individually (Fig. 4).

Cell-free *F. nucleatum* membranes are sufficient to induce cell death in Jurkat cells. To further characterize the nature of cell death induction by the membrane proteins Fap2 and RadD, we examined if their function depended on the presence of other fusobacterial components. To accomplish this, we isolated membranes from wild-type *F. nucleatum* and the respective *fap2*, *radD*, and *fap2 radD* mutant strains for incubation with Jurkat cells. Wild-type *F. nucleatum* membranes induced cell death in Jurkat cells at levels similar to that produced by incubation in the presence of whole cells. Therefore, the killing factor was likely present in the *F. nucleatum*-enriched membrane fraction and did not require an ATP-dependent process (Fig. 2). Jurkat cells incubated with *fap2* or *radD* mutant membranes demonstrated partial decreases in cell death of 51% and 27%, respectively, while incubation with the *fap2 radD* mutant membranes resulted in a 77% decrease in cell death (Fig. 2). This decreased cell death rate in the presence of *fap2*, *radD*, or *fap2 radD* membranes exhibited a trend similar to that for whole cells. Similar experiments performed with membranes isolated from the mutant derivative lacking AimI induced cell death in Jurkat cells similarly to the wild type (data not shown), further indicating that this protein is not part of the same mechanisms represented by Fap2 and RadD. Taken together, these findings further support the idea that the membrane portion of *F. nucleatum* alone, specifically the outer membrane proteins Fap2 and RadD, is responsible for the induction of cell death in lymphocytes.

DISCUSSION

F. nucleatum is one of the most abundant microorganisms isolated from healthy and diseased patients, so its association with periodontal disease (34, 35) has been tenuous and often attributed to its co-occurrence with pathogenic species that are not normally present in healthy patients and possess known virulence factors (27). Our previous findings strongly implied an involvement of *F. nucleatum* in the development of periodontal disease through suppression of the immune system and induction of cell death in lymphocytes mediated by a heat-labile pronase-sensitive cell surface protein(s) (16). Here we provide evidence that the outer membrane proteins Fap2 and RadD of *F. nucleatum* have a role in triggering cell death events in Jurkat cells. Fap2 and RadD are classified as members of the type Va autotransporter family, and we suspect that they may induce cell death via a contact-based mechanism rather than through secreted effector proteins although the exact mechanism through which these proteins induce cell death is currently under investigation. This mechanism would be in contrast to those of other autotransporter proteins implicated in eukaryotic cell death, which are secreted into the cells (5) or function as proteases (40). Most importantly, these findings have important implications for the perceived pathogenic nature of *F. nucleatum*, specifically, for the mechanism it uses to suppress the immune system, persist in the oral cavity, and contribute to disease. Further understanding this system could enable the development of alternative approaches to control the pathogenesis of *F. nucleatum* and treat periodontal disease.

A significant amount of research has been performed in bacteria regarding the role of cell death in their virulence. The majority of mechanisms discovered to date employ small proteins and secretion systems that transport effector proteins into the host cell (8). In contrast, the evidence presented here indicates that the outer membrane proteins of *F. nucleatum* identified here act alone to induce cell death (Fig. 2). Inactivation of all *fap2* homologs present in the transformable *F. nucleatum* strain ATCC 23726 led to the identification of Fap2 and RadD as the proteins primarily responsible for the induction of cell death (Fig. 2). The previously described Fap2 homolog AimI (22) was associated with a partial decrease in the ability to induce cell death in Jurkat cells. However, since membranes of the corresponding mutant strain induced cell death in Jurkat cells at levels similar to those observed for wild-type membranes, the underlying mechanism is clearly different from that for Fap2 and RadD. Furthermore, inactivation of *aim1* did not result in a further decrease in cell death induction when combined with *fap2* and *radD* mutations (data not shown). While individual gene inactivation mutations in *fap2* and *radD* resulted in a considerable reduction in cell death induction, the corresponding double gene inactivation mutant almost completely lacked this ability, suggesting that both proteins play important roles in *F. nucleatum* virulence (Fig. 2). The redundancy of the cell death signals generated by these two *F. nucleatum* proteins seems contradictory to the supposed compact and streamlined nature of bacterial genomes, especially considering the sizes of the proteins coded by these genes. Since both proteins are present in the membrane, they may associate and form a complex that more efficiently induces cell death. However, it is clear from our results that such a complex is not necessary, as each protein alone is sufficient to induce cell death, albeit at a lower level (Fig. 2). Each protein appears to have a yet-unknown role in the process of inducing cell death, and the combination of their respective activities is required for *F. nucleatum* to trigger cell death in lymphocytes most efficiently. Clearly, further experiments are necessary to unravel the detailed function of each protein in this process.

Fap2 and RadD are homologous to autotransporter proteins, and the recent finding that most passenger domains are β-helical structures appears to be true for *F. nucleatum* (18, 31). Analogous to other proteins with passenger domains, Fap2 and RadD have little similarity to each other outside their translocator domains. The large size of the passenger domain allows for a multifunctional protein containing several active sites involved in multiple interactions with the host cells. In *Bordetella pertussis* the membrane-associated and secreted filamentous hemagglutinin (FHA) facilitates the adherence of bacteria to host cells, may help determine host specificity (15), and possesses at least four different binding domains conferring separate attachment specificities (28, 31). Similarly, *F. nucleatum* adheres to bacteria and host cells using a different adhesin, including the autotransporter RadD, which, based on the results presented in this study, has an additional function in cell death induction (11, 36, 43). *F. nucleatum* may use its ability to adhere to lymphocytes to help it induce cell death and deplete the immune system in the local area. The adhesion function of RadD would at the same time facilitate colonization of this area by enabling attachment of other bacterial species. In addition, Fap2 and other outer membrane proteins in *F. nucleatum* may possess adhesin domains that allow adherence to different groups of bacteria and host cells. While the *F. nucleatum* galactose-inhibitable adhesin has not yet been identified, extensive biochemical analyses demonstrated its presence in the membrane (36). By modulating the expression of its numerous T5SSs, *F. nucleatum* could shape its environment by adhering to specific surfaces, suppressing immune function, and recruiting other bacteria to the oral biofilm. Future work will further characterize these outer membrane proteins and possibly reveal the presence of specific domains that are associated with adhesion and cell death induction. Understanding how these proteins function will further our understanding of the pathogenesis of *F. nucleatum* and may offer insight into the pathogenesis of other species employing similar mechanisms.

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